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Uptake of Carboxyfluorescein-Containing Liposomes by Peyer's Patch and Non-Peyer's Patch Tissue of the Rat Intestine

by

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13. ABSTRACT *(Maximum 200 words)*

Studies have shown that stress in soldiers caused by caloric and sleep deprivation coupled with performance anxiety can markedly suppress immune function. This study was designed to test the possibility of oral targeting of liposomes containing immune-enhancing cargos to the Peyer's patch follicles of the small intestine. The mucosal immune system is the body's primary defense against the entry of infectious agents from the intestinal lumen, and Peyer's patches (PP) are the specific lymphoid follicles located along the small intestine. This report contains the results of three studies on rats orally intubated with immune system-targeted liposomes stabilized against gastrointestinal stresses and containing a fluorescent marker, 5(6)-carboxyfluorescein (CF). In the study showing the greatest uptake, one hour after intubation, saline wash showed that 40% of liposomes fed had remained intact in the gut. In this study, total liposome CF uptake per rat was 0.12% of dose, three times that of free CF. In the 45 cm intestinal segment with the most active uptake, specific uptake of liposomal contents in PP was 0.470 nmol/cm2, compared to non-Peyer's patch (NP) of 1.64 nmol/cm2. The specific uptake of liposomal contents in PP was 3.76 times that previously reported (Tomizawa, et al.), although lipid uptakes were similar. This result is partly because we achieved a 4.5 fold greater cargo loading of our liposomes. These uptakes are adequate for amplifying compounds like antigens and hormones but seem insufficient for targeting immune-enhancing nutrients. Chronic rather than single dose administration and more highly loaded liposomes or latex microspheres may improve uptakes.

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PREFACE

This study on oral targeting of liposomes to the Peyer's patch follicles of the small intestine was a cooperative project of the U.S. Army Natick Research, Development and Engineering Center and the U.S. Army Research Institute of Environmental Medicine. It was carried out between 9 June and 30 August 1995, under funding number AH-5261. Grateful acknowledgement is made for the able critiques of Dr. K. A. Narayan and Dr. W. D. Bowers, painstaking editorial improvement of the manuscript by Marcia Lightbody, and the technical assistance of SGT A. I. Osagie.

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as official Department of the Army position, policy or decision, unless so designated by other official documentation. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services, NIH Publication No.86-23, revised 1985, as prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources Commission on Life Sciences, National Research Council. United States Army Research Institute of Environmental Medicine is an AAALAC approved facility and will continue to adhere to the standards and requirements thereof. Citations of commercial organizations and trade names do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

EXECUTIVE SUMMARY

The immune system of stressed soldiers is often compromised. The mucosal immune system is the body's primary defense against the entry of infectious agents from the intestinal lumen, and Peyer's patches (PP) are the specific lymphoid follicles located along the small intestine. This report concerns the results of three studies on rats orally intubated with immune system-targeted liposomes stabilized against gastrointestinal stresses and containing a fluorescent marker, 5(6)-carboxyfluorescein (CF). In the study showing the greatest uptake, one hour after intubation, saline wash showed that 40% of liposomes fed had remained intact in the gut. In this study, total liposome CF uptake per rat was 0.12% of dose, three times that of free CF. In the 45 cm intestinal segment with the most active uptake, PP specific uptake of liposomal contents was 0.470 nmol/cm^2 compared to non-Peyer's patch (NP) of 1.64 nmol/cm^2. The PP specific uptake of liposomal contents was 3.76 times that previously reported by Tomizawa, et al. (Pharm. Res. 10, 549, 1993), although lipid uptakes were similar. This is partly because we achieved a 4.5 fold greater cargo loading of our liposomes. These uptakes are adequate for amplifying compounds like antigens and hormones but seem insufficient for targeting immune-enhancing nutrients. Chronic rather than single dose administration and more highly loaded liposomes or latex microspheres may improve uptakes.

UPTAKE OF CARBOXYFLUORESCEIN-CONTAINING LIPOSOMES BY PEYER'S PATCH AND NON-PEYER'S PATCH TISSUE OF THE RAT INTESTINE

I. INTRODUCTION

a. Objective: The objective of this report is to summarize results of three studies in orally intubated rats of fluorescent cargo-loaded liposome uptake by the intestinal immune system, the Peyer's patches (PP).

b. Purpose of Study: Studies at the U.S. Army Ranger Training Course (Shippee, et al., 1994) have shown that stress by caloric and sleep deprivation, coupled with performance anxiety, can markedly suppress immune function. This fact, together with potential contact with exotic diseases, poses a threat to OCONUS operations. This study was designed to test the possibility of oral targeting of liposomes containing immune-enhancing cargos to the PP follicles of the intestine, the gut associated lymphoid tissue (GALT, Krammer, H.-J. and Kuhnel, W.,1993), one form of the mucosal associated lymphoid tissue (MALT) which includes lung, nasal and buccal epithelium. There is evidence that enhanced immunity developed in the gut is relayed to other MALT, such as lung (Ruedl, C. et al.,1993). Potential immune-enhancing compounds include vitamin E, vitamin C and beta-carotene, all of which have been shown by us to be very easily incorporated as liposome cargos. It is probable that the highly potent immune enhancing amino acid, glutamine, can be similarly incorporated. Liposomes with immune target affinity (large size and negative charge), in addition to gastrointestinal stability (high cholesterol and long lipid chains) have been developed, and were used in this study. Two recent studies (Aramaki, et al., 1993 and Tomizawa, et al., 1993) have confirmed that such large negatively charged liposomes are actively accumulated by PP.

II. MATERIALS AND METHODS

a. Materials: L-alpha-distearoylphosphatidylcholine (DSPC, 99% purity), L-alpha-dipalmitoylphosphatidyl-L-serine (DPPS), cholesterol (C, 99% purity) and isoamyl alcohol (98.5% purity) were procured from Sigma Chemical Co. (St. Louis, MO). Tert-butanol was procured from Fisher Scientific Co. (Pittsburg, PA). 5(6)-carboxyfluorescein (CF, 99% purity) was purchased from Eastman Chemical Co. (Rochester, NY).

b. Preparation of Liposomes: A mixture of lipids (10 umol DSPC, 10 umol DPPS, and 20 umol C) was dissolved in 2 mL t-butanol, at 75°C (above the lipid transition temperature). The solution was evaporated to a thin film on a Buchi Rotovapor R and dispersed in 1 mL of phosphate buffered saline (PBS, pH 7.4)

containing 84 mM CF at 75°C, using a Vortex-Genie Mixer (5 cycles of 20 sec each). The dispersion was then subjected to five freeze-thaw cycles, in order to distribute the CF among the multilayers and maximize the trapped volume (Mayer, et al., 1986). The CF-loaded liposomes were extruded 10 times through two stacked 0.4 μm pore polycarbonate membrane filters (Poretics, Inc.) at 75°C using a Thermobarrel Lipex Extruder (Lipex Biomembranes, Inc.) under N_2 gas pressure at 100-200 psi. It was found necessary to substitute fresh filters after the first extrusion and to use t-butanol rather than chloroform/methanol as initial solvent to avoid cholesterol microcrystallization in the membranes, which clogs the filter, as has been reported in the Lipex manual. Finally, nonencapsulated CF was removed using PBS buffer as eluant on a Sepharose 4B-200 column (1 X 19 cm, Sigma Chemical Co.), resulting in about a 3/1 dilution. Photometric measurement of final concentration of CF and estimate of lipid concentration by scatter profile (Whitburn and Dunne, 1994) was performed on a Gilford Response UV/VIS Spectrophotometer (Gilford Systems, Oberlin,Ohio). At 84 mM, CF undergoes quenching of fluorescence and the liposome dispersions are opalescent orange, whereas the expected fluorescence is observed upon detergent breaching. We have shown that the fluorescence has a nearly linear dependence on concentration below 10 uM. Details of loading and feeding conditions appear in Table 1.

c. In vivo Procedures for Rat Oral Administration and Surgical Recovery of Liposomes: Three experiments, denoted RATIV, RATV and RATVI, were carried out. Details of loading and feeding are contained in Table 1. In a typical experiment, two rats (Sprague-Dawley, 380-440 g, varying in age from 85-93 days) were fasted for 24 hours. A measured 5 mL of liposome suspension (630 uM in CF and ca. 5.9 mM in lipid) was administered by gavage to one rat. Dissolved free CF in 5 mL PBS (630 uM) was administered to the other rat. After 1 h, each rat was anaesthetized with methoxyflurane, the diaphragm was cut, and the small intestine isolated. It was ligated and cut at the end of the duodenum and at the entrance to the caecum. The ends were labeled. Saline (100 mL) was flushed through in 30 mL portions. The effluent was saved for analysis. The intestine was cut into 15 cm segments and labeled in order from the duodenum end. Peyer's patches (PP, easily identified with the naked eye) from each of the segments were excised as a circular piece of uniform size (28 mm^2). For each piece of PP, an adjacent equal area non-Peyer's patch piece (NP) was cut. All the PP pieces from one segment were placed in a microcentrifuge tube containing 1 mL saline. The non-PP pieces were similarly collected, segment by segment. Subsequently, pieces from several segments were collected into tissue groups as shown in Table 2, to facilitate analysis.

d. Analysis of Saline Wash and Tissue Distribution: A total of six rats were used for these experiments (two per experiment). The available tissues were divided into three groups, as shown in Table 2. The first three 15 cm segments were combined into Group I; the 4th-6th 15 cm segments were combined into Group II; and the remainder of the intestine was Group III.

TABLE 1
Liposome Loading and Feeding Conditions and Specific Uptake
Rat Oral Liposome Study

Ref. # or Natick study	Marker Conc. in Liposomes µM	Lipid Conc. in Liposomes mM Ratio[a]	Marker Fed/rat nmol	Lipid Fed/rat µmol	Highest Marker Uptake pmol/cm^2	Highest Lipid Uptake/ nmol/cm^2
Ref. 1	RhPE[b] 136	33 ratio 243	136	33	PP 155	38
Ref. 8	CF 24	1 ratio 42	24	1	PP 125	5.2
Rat Study IV	CF 630	5.9 ratio 9.4	3150	29	NPLII[c] 1640 PPLII 470	PPLII 4.4
Rat Study V	CF 630	5.9 ratio 9.4	2520	24	PPFII 249	PPFII 2.3
Rat Study VI	CF 386	3.8 ratio 9.9	1930	19	PPFII 381	PPFII 3.8

a Ratio of lipid to marker.
b RhPE is rhodamine B-phosphatidyl ethanolamine
c PP is Peyer's Patch; NP is non-Peyer's patch tissue from an area adjacent to the PP sample. FI, FII, and FIII are the Groups I, II and III tissues from rats fed free CF; LI, LII, and LIII tissues are from rats fed liposome encapsulated CF. See Table 2.

e. Analytical Procedures - Saline Wash: The 100 mL of saline and intestinal contents flushed through the small intestines were thoroughly mixed and a 1 ml aliquot was mixed with 4 mL normal saline and centrifuged 5 min on an International Clinical Centrifuge. A clear supernatant resulted. Fluorescence intensity was measured in the right angle mode using a SPEX Fluorolog 2 Spectrofluorometer (Edison, NJ) in the photon counting mode. The instrument has a 450 watt Xenon lamp and an emission voltage of 900 V with reference voltage of 400 V was employed, with entrance slits 0.1, 0.1 and emission slits 2.0, 1.5 mm with 1 cm quartz cuvettes. Excitation was set at 492 nm and emission intensity was recorded, normally at about 516 nm. Intensity was again recorded after addition of 1 mL of 12.5% Triton X-100 to 2 mL of the supernatant, to breach the liposomes.

Table 2
Characteristics of Tissue Groups from Rats Fed Liposomes and Free Solution
Length and Number of Tissues in Tissue Groups

Group	Liposome			Free CF		
	Length	# PP	# NP	Length	# PP	# NP
RAT STUDY IV						
I	45cm	PPLI[a] 6	NPLI 6	45 cm	PPFI 3	NPFI 3
II	45	PPLII 6	NPLII 6	45	PPFII 3	NPFII 3
III	20	PPLIII 4	NPLIII 4	15	PPFIII 3	NPFIII 3
RAT STUDY V						
I	45 cm	PPLI 4	NPLI 4	45 cm	PPFI 4	NPFI 4
II	45	PPLII 4	NPLII 4	45	PPFII 7	NPFII 7
III	41[b]	PPLIII 7	NPLIII 7	14	PPFIII 3	NPFIII 3
RAT STUDY VI						
I	45 cm	PPLI 5	NPLI 5	45 cm	PPFI 10	NPFI 10
II	45	PPLII 5	NPLII 5	50	PPFII 12	NPFII 12
III	30	PPLIII 4	NPLIII 4	[c]		

a This intestine was suspended during cutting which resulted in stretching.
b Short intestine; no group III for this animal.
c See Table I for group abbreviations.

f. Analytical Procedures - Tissue: Tissues from one tissue group (Table 2) were placed in 2 mL of normal saline and homogenized 1 min by Polytron (Kinematica Instruments, Inc., Lucerne, Switzerland). The sample was next heated at 60°C for 30 min with 200 ul of Triton X-100 to breach the liposomes. One ml of 1 N HCl and six ml of isoamyl alcohol were added, the sample mixed by 20 inversions and 30 sec vortexing. Five min centrifugation on the International Clinical Centrifuge usually separated the layers. To five ml of the isoamyl top layer, five mL of bicarbonate buffer (10 mM, pH 10) were added and mixing, clarification and fluorescence intensity measurement were performed as above on the buffer layer.

g. Analytical Procedures - Fluorescence-Concentration Correlation: Using a molar extinction coefficient of 60, solutions of CF of known concentration $\leq$ 100 uM were prepared. It was found that quenching begins at about 10 uM, but below 1 uM, there is a nearly linear dependence of concentration on fluorescence intensity. A

reading of 1000 photons per second corresponds to 0.0125 uM. All readings, including that of the saline flush, were in this intensity region.

h. Analytical Procedures - Recovery Studies: Studies of recovery of 880 nmol of CF in 0.5 mL PBS incubated as long as 64 hours with three excised PP or three NP tissues showed only 0.004% recovery after 12 5-mL washings with Milli-Q water and the standard extraction procedure above. On the other hand, recovery studies of 20 ul of 176 uM CF (3.5 nmol) added to 2 mL Milli-Q water and given the standard extraction procedure, showed a mean of 88% recovery in two trials. It is estimated that the rat intestine has a volume, undistended, of 5-7 mL, so a 100 mL flush is equivalent to 15 to 20 5-mL washes. Thus, although there is good recovery of free CF from solution by the standard extraction procedure, tissues incubated with free CF in vitro after removal from the animal and given washes comparable to the saline flush show very little uptake or contamination.

III. RESULTS

a. Saline Flush: Study RATIV showed the most definitive results from the saline wash, as shown in Table 3.

Table 3
Recovery of CF in 100 mL Saline Flush in Study RATIV

	Free CF	Liposomal CF
Pre-Triton Concentration	14.5 µM	6.5 µM (apparent)
Pre-Triton Amount	1.45 µmol	0.65 µmol (apparent)
% of Original Load[a]	46	21 (apparent)
Post-Triton Concentration[b]	12.0 µM	11 µM
Post-Triton Amount	1.2 µmol	1.1 µmol
% of Original Load	38	35

a From Table 1
b Corrected for dilution by Triton

These results show that almost half of the free CF dose fed was recovered in the flush, whereas only 21% apparently remained of the liposome dose. However, Triton breach showed that actually about 35% of the fed liposome dose still remained, 14% in the fluorescence quenching, liposome-contained mode that would have been

available for Peyer's patch uptake. Studies RATV and RATVI showed somewhat less recovery and less evidence for liposome stability in the gut.

b. Tissue Analysis - Specific Vs. Total Uptake: Uptake can be expressed as specific uptake (amount/cm^2) or total study uptake, which for PP equals projected total gut PP uptake, since all detectable PP tissue was assayed. However, for NP, total study uptake must be multiplied by an areal adjustment factor to obtain projected total gut NP uptake, since only between 2.5- 4% of total NP intestinal area was sampled. This is a rough approximation, but is justified since Tomizawa (1993) found NP uptake independent of intestinal location.

Tissue groups were analyzed for concentration in the final bicarbonate buffer and from this was calculated the amount per group (e.g. mol/PPLI). This was converted to amount per tissue (PP or NP), by division by the number of tissues per group. Multiplication by (100 mm^2/28 mm^2) gave amount per cm^2, which has been used by previous workers (Tomizawa, et al., 1993) as a measure of specific uptake. Lipid concentration in the fed dose and specific lipid uptake/cm^2 were estimated from amount of initial lipid carried through to the fed dose and its ratio to the known CF concentration, assumed constant after gel filtration. These data by group are shown in Table 1 and Figures 1-3. Table 1 and Fig. 1 show that our best Peyer's patch liposome specific uptake of lipid (Study RATIV, PPLII, 4.4 nmol/cm^2) is similar to that of Tomizawa (5.2 nmol/cm^2), but our specific uptake of CF is 3.8 times as great (PPLII, 470 vs. 125 pmol/cm^2, Tomizawa). This is probably because we achieved a 4.5 fold greater cargo loading factor in our liposomes and a 130 fold greater CF concentration in the gut at intubation. Note that intestinal segments in Group II, the more distal 45 cm, consistently (with one marginal exception) had the greatest specific uptake, whether liposome or free, PP or NP. This finding is similar to the findings of Tomizawa, et al (1993) and Aramaki, et al. (1993), which showed greater specific uptake in the lower ileum.

Total study uptake and area adjusted total uptake by both tissue and vehicle type is shown in Figures 4 and 5. The area adjustment factor (AAF) is as follows:

$$AAF = ((MA - (\#PP \times 0.28\ cm^2)) / (\#PP \times 0.28\ cm^2)$$

Where:
MA = Measured intestinal length x 1 cm (estimate of internal intestinal circumference) and NP area = PP area.

Breakdown of total uptake by tissue type is shown in Figures 6 and 7, and total by vehicle type in Figures 8 and 9. Uptake as percent of fed dose for liposomes vs free-fed is shown in Figure 10. In the most successful experiment (RATIV), total assayed liposome marker uptake was 0.12% of fed load (Fig.8 and Table 1), but, when projected to total intestinal area, was 2.3% (Fig. 10). This was 3.2X (unprotected, Fig.8) and 3.3X (projected, Fig. 9) that of free marker uptake. Data from

Fig. 8 and Table 1 also show that in RATIV, total uptake of combined free and liposome fed load was 0.16%, but when projected to full area was 3% (Fig. 10). In RATV and RATVI, free marker uptake exceeded liposome loaded. In RATIV, PP unprotected uptake was half that of NP unprotected (Fig. 6), and when corrected for area, was only 2% of NP uptake (Fig. 7). In no case was a strong excess of PP vs NP uptake (unprotected) noted as found by Tomizawa, et al. (1993) and Aramaki, et al. (1993).

IV. DISCUSSION

Although we achieved specific liposome-loaded cargo uptakes (amt/cm^2) much greater than those of previous workers (Tomizawa, et al. 1993, and Aramaki, et al., 1993) we could not demonstrate a consistently greater specific uptake for liposome-loaded cargo over that in free solution. In five out of six of the more distal groups from each study (Groups II, the groups that were noted to have the highest specific uptake), PP uptake exceeded NP, but not always by a great margin, and with the exception that the highest specific uptake observed was in NPLII, not a PP group. Thus, except for the finding that the more distal segments had the highest specific uptake, our results suffer from high variability and do not support the findings of the Japanese workers.

The reasons for these discrepancies are not clear. We used a circular sample with a standard area, which may have often included some NP tissue when sampling a small PP. Number of available PP (and hence the companion NP) tissues varied widely, although our calculation of specific uptake per unit area and area adjusted total uptake should normalize the effect of this variation. In addition, liposome size is known to affect uptake, as Tomizawa (1993) showed. However, he found that extrusion in his manner and as we performed it, is considered to produce quite homodisperse liposomes of about 400 nm diameter. We employed single dose administration and one hour sampling. It seems clear from the work of others with particle uptake by PP (LeFevre, et al., 1989), that chronic rather than bolus feeding gives more uptake and less variability. Total liposome-loaded cargo uptake was only 0.12% of fed load and rose to only 2.3% when adjusted for the additional unsampled NP area. At our cargo loading, we fed 1.2 mg in Study IV, and achieved a possible 36 ug combined free and liposome uptake. Clearly, except for effect-amplifying cargoes like antigens or hormones, this is insufficient for practical immune-enhancing nutrient supplementation (Souba, 1993).

VI. CONCLUSIONS

Three studies were conducted with rats orally intubated with immune system-targeted liposomes stabilized against gastrointestinal stresses and containing a fluorescent marker, CF. In the most productive study, one hour after intubation, saline wash showed that 40% of liposomes fed had remained intact in the gut. In this study,

total liposome CF uptake per rat was 0.12% of dose, three times that of free CF. In the group representing the 45 cm segment with the most active uptake, PP specific uptake of liposome marker was 0.470 nmol/cm^2 compared to NP of 1.64 nmol/cm^2. The PP liposome marker specific uptake was 3.76 times that previously reported by Tomizawa, et al. (1993), although lipid uptakes were similar. This is probably caused by our 4.5 fold greater liposome cargo loading. These uptakes, though much greater than those of the previous workers, are adequate for effect-amplifying compounds like antigens and hormones, but seem insufficient for targeting immune-enhancing nutrients. Chronic rather than single dose administration and more highly loaded liposomes or latex microspheres may improve uptakes.

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VII. FIGURE LEGENDS

Figure 1-3 PP is Peyer's Patch; NP is non-Peyer's patch tissue from an area adjacent to the PP sample. FI, FII, and FIII are the Groups I, II and III tissues from rats fed free CF; Groups LI, LII, and LIII tissues are from rats fed liposome encapsulated CF.

Figure 4 Same as above combined for RATIV, V, and VI.

Figure 5 Area Adjusted, see text for area adjustment formula, abbreviations same as above.

Figures 6-10 Use same abbreviations as above.

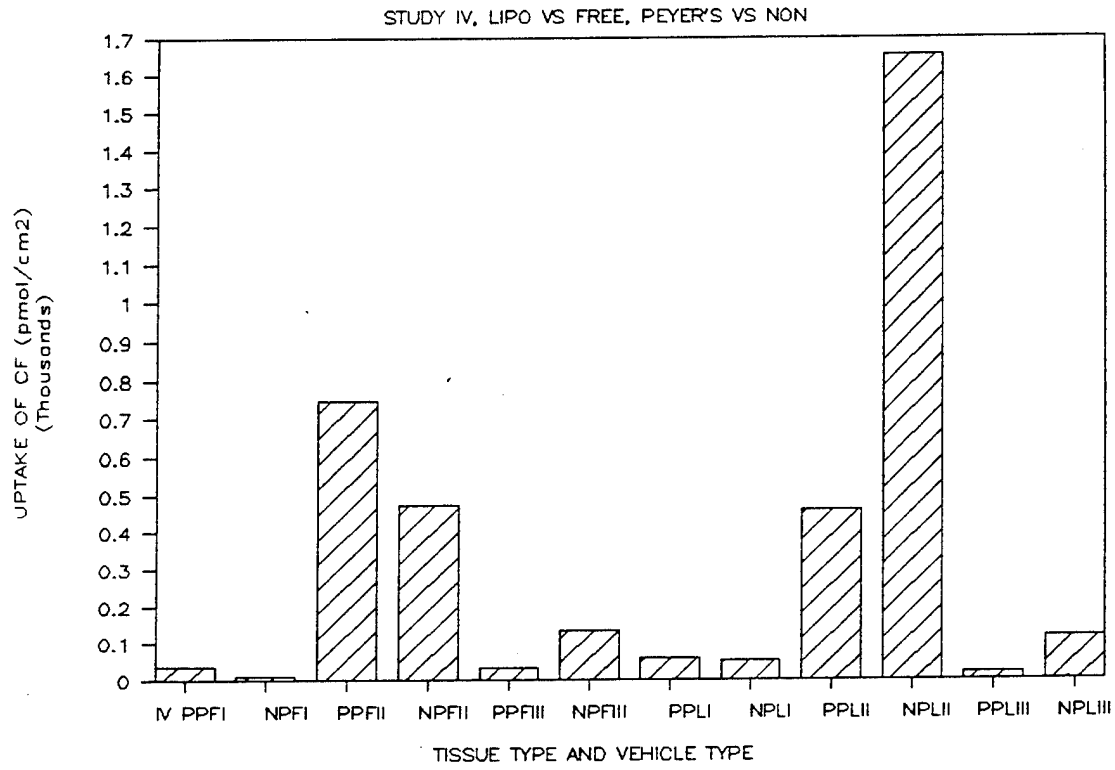

Figure 1. Specific Uptake of Liposomes By Tissue Groups Exp. IV

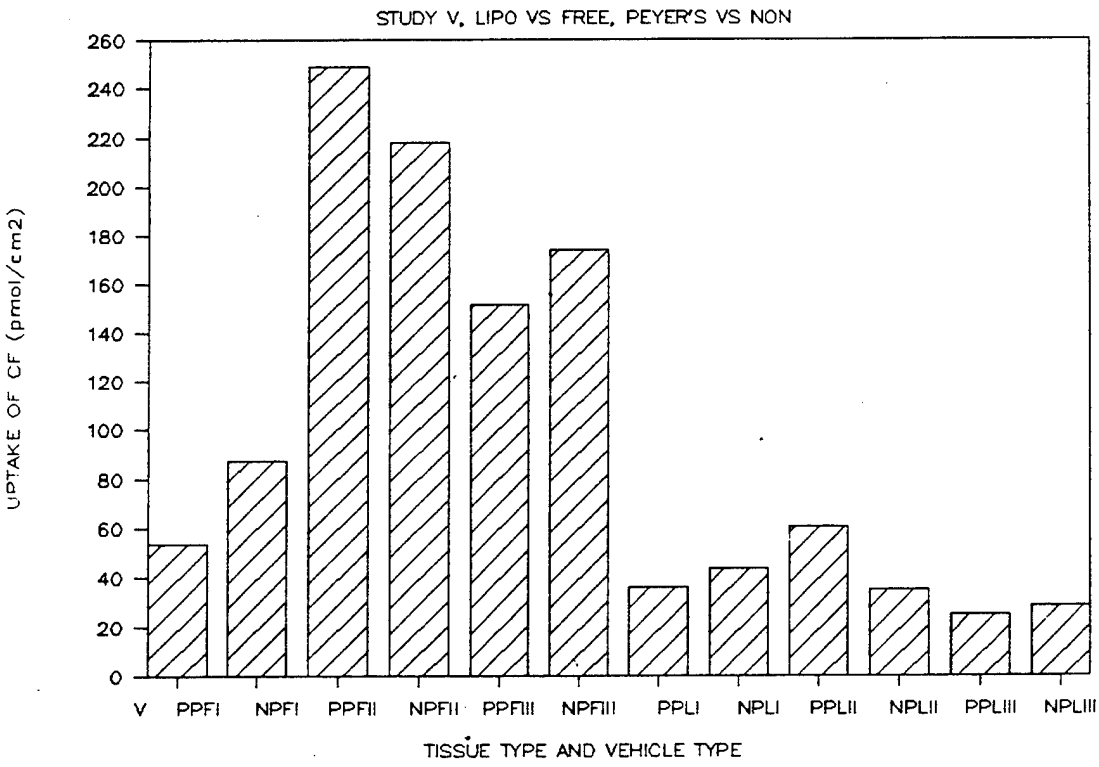

Figure 2. Specific Uptake of Liposomes By Tissue Groups - Exp. V

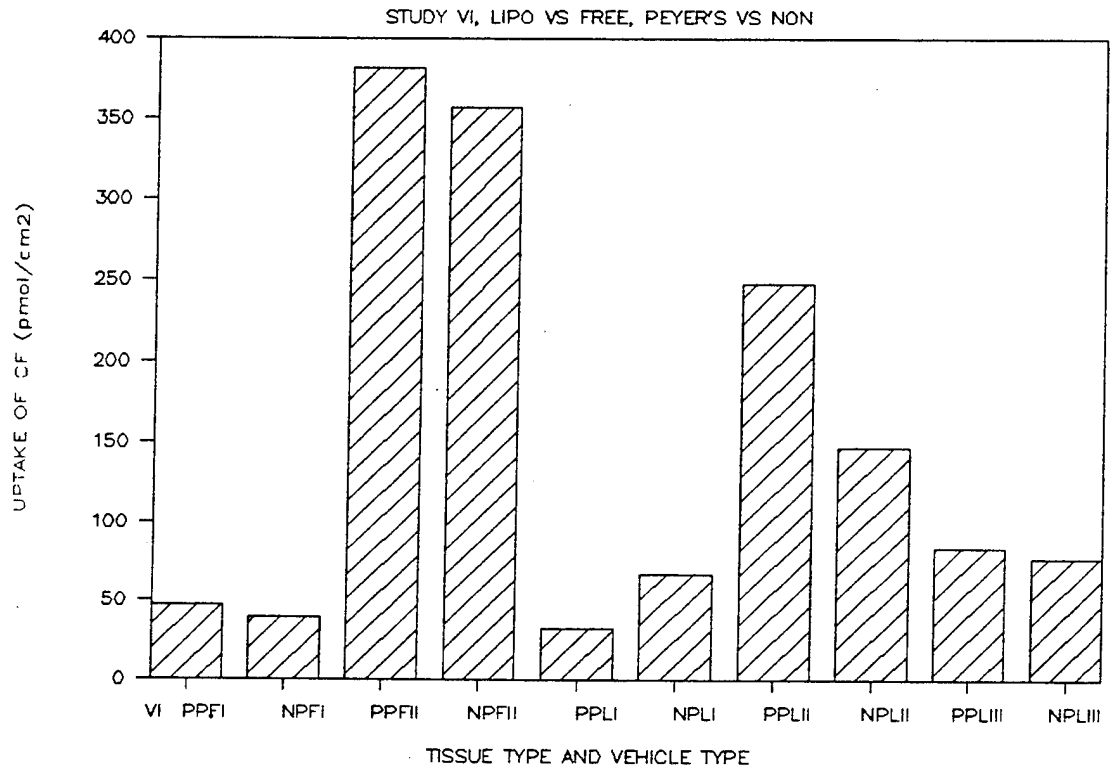

Figure 3. Specific Uptake of Liposomes By Tissue Groups - Exp. VI

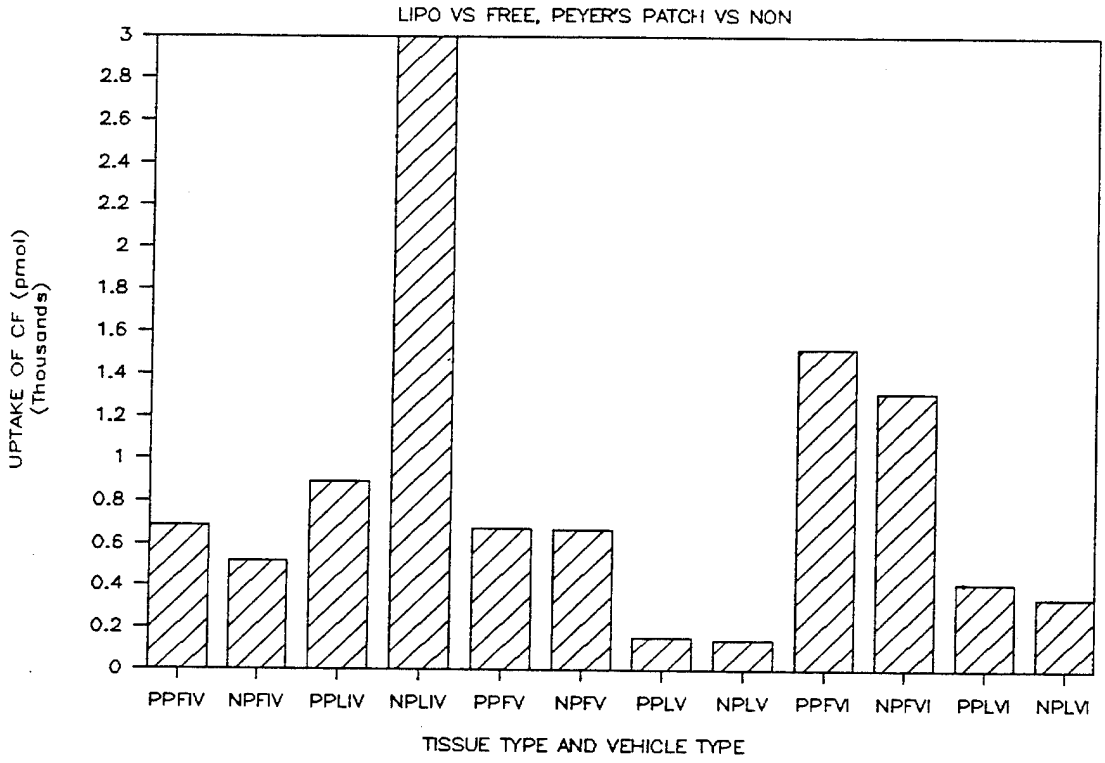

Figure 4. Total Uptake By Tissue Type and Vehicle Type

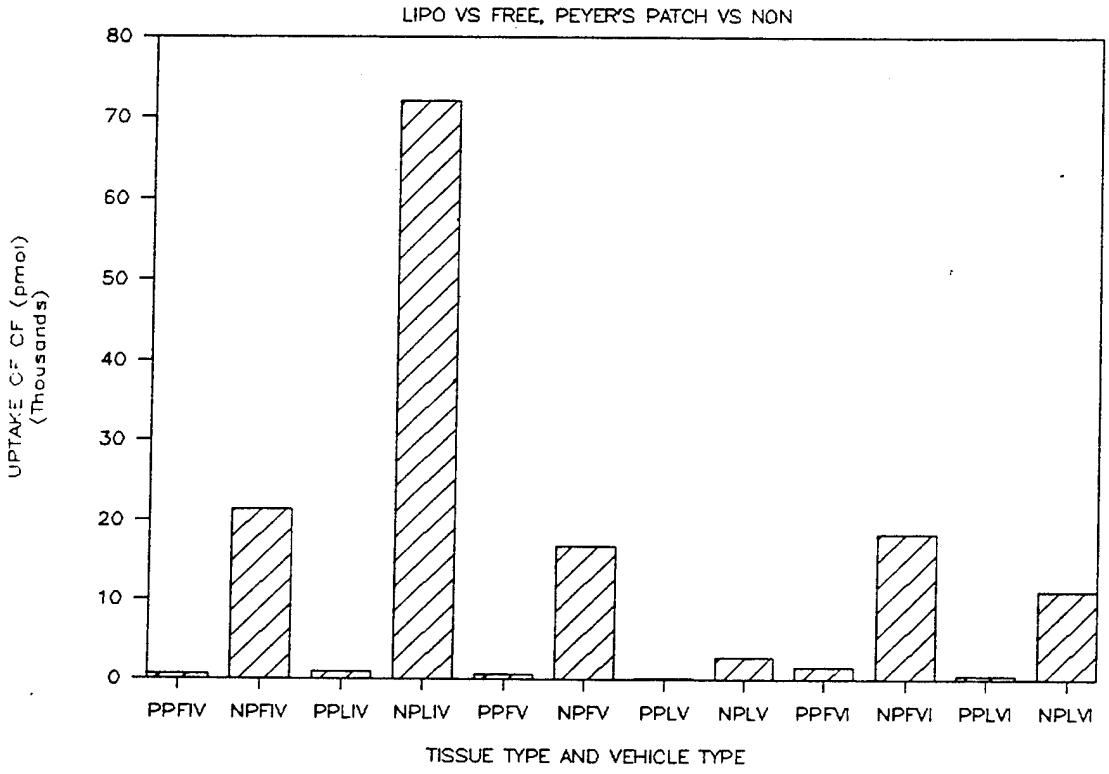

Figure 5. Total Uptake By Type Adjusted for Area

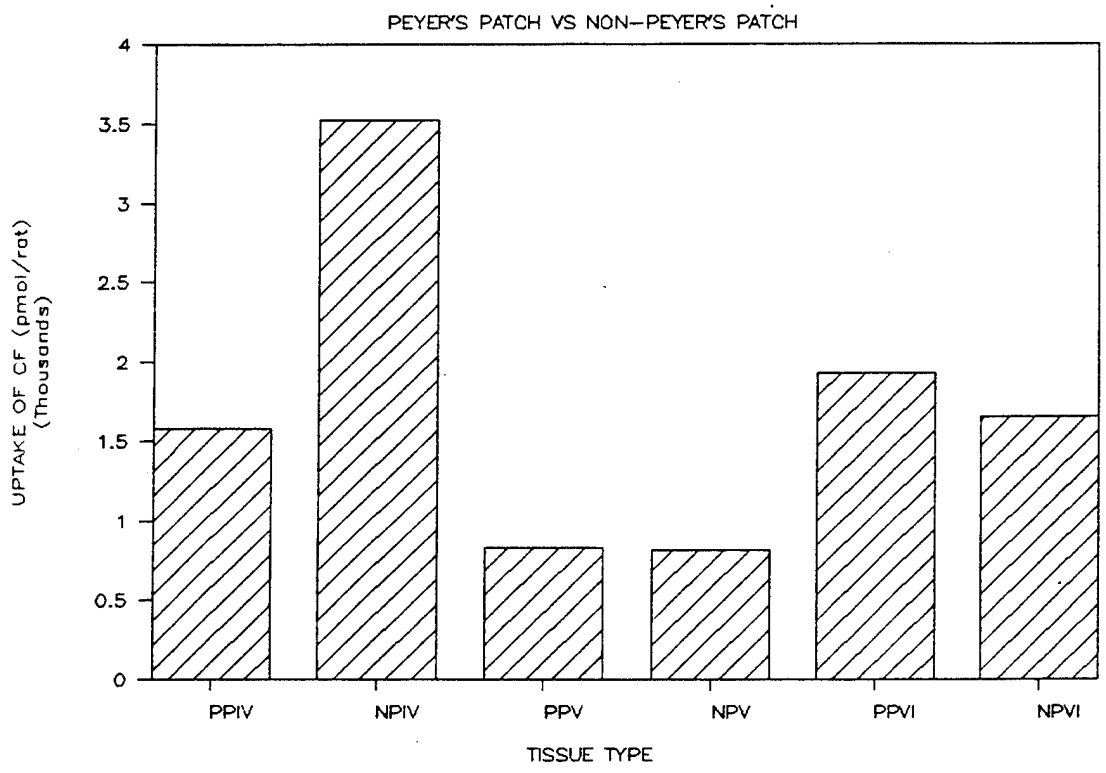

Figure 6. Total Uptake Per Rat - Peyer's Patch (PP) vs. Non-Peyer's Patch (NP)

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